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ANTHOCYANINS FROM FLOWERS AND LEAVES OF *NYMPHAÉA* × *MARLIACEA* CULTIVARS

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Key Word Index—*Nymphaéa* × *marliacea*; Nymphaéaceae; flowers; leaves; anthocyanins; diacylation; cyanidin 3-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-β-galactopyranoside); ^{13}C-NMR; chemotaxonomy.

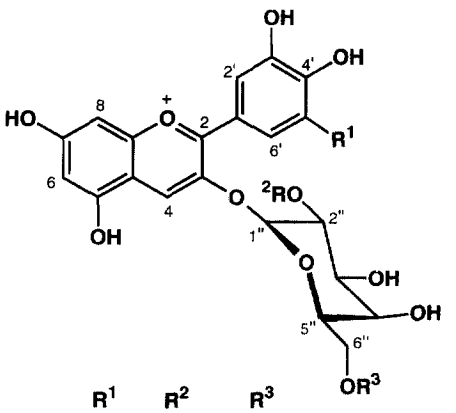
Abstract—Five anthocyanins have been isolated from red flowers of the water lily, *Nymphaéa* × *marliacea* var. Escarboucle and identified by a combination of chromatography, electrospray MS and homo- and heteronuclear two-dimensional NMR techniques to be the novel pigment cyanidin 3-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-β-galactopyranoside), and the 3-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-β-galactopyranoside), 3-*O*-(6″-*O*-acetyl-β-galactopyranoside), 3-*O*-(2″-*O*-galloyl-β-galactopyranoside) and 3-*O*-β-galactopyranoside of delphinidin. The same five anthocyanins were also found in different proportions in both flowers and leaves of two other *N.* × *marliacea* cultivars with pink and white flowers, respectively. The chemotaxonomic significance of the acylated delphinidin derivatives is discussed.

INTRODUCTION

As part of our work on colour variation due to acylated anthocyanins, we have recently reported two novel delphinidin derivatives from leaves of the water lily (*Nymphaéa* × *marliacea*) cultivar with white flowers [1]. The major pigment, delphinidin 3-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-β-galactopyranoside), contained gallic acid, which seems to have a very restricted distribution as acylation agent of anthocyanins [2–4]. Hence, this paper deals with the identification of the qualitative and relative quantitative anthocyanin content in both flowers and leaves from three cultivars of the water lily, *Nymphaéa* × *marliacea*, with different flower colours.

RESULTS AND DISCUSSION

The HPLC chromatogram of the methanolic extract from red flowers of *Nymphaéa* × *marliacea* var. Escarboucle detected in the visible spectral region showed three major anthocyanins, **3**–**5**, in addition to four minor anthocyanins (together 4%) (Table 1). Pigments **1**–**5** were purified by partition against both hexane and ethyl acetate followed by Amberlite XAD-7 column chromatography. The pigments were separated by preparative HPLC. The pure anthocyanins were checked for homogeneity by analytical HPLC and TLC (Table 2).



	R^1	R^2	R^3
1	OH	H	H
2	OH	galloyl	H
3	OH	H	acetyl
4	OH	galloyl	acetyl
5	H	galloyl	acetyl

Compounds **1**, **3**, **4**, were identified as the 3-*O*-β-galactopyranoside, 3-*O*-(6″-*O*-acetyl-β-galactopyranoside) and 3-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-β-galactopyranoside) of delphinidin, respectively, by means of UV–Vis spectroscopy, electrospray MS, chromatography (Table 2), and homo- and heteronuclear

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Table 1. Relative proportions (%) of the anthocyanins in flowers and leaves of three cultivars of *Nymphaéa* × *marliacea*

	Pigments[a]					
	1	**2**	**3**	**4**	**5**	Unknown
Cultivar with red flowers (var. Escarboucle)						
Whole flowers	2	1	23	56	15	3
Petals	3	3	24	59	11	t
Stamens	2	3	26	47	21	1
Leaves	t	2	5	74	16	3
Cultivar with pink flowers						
Whole flowers	4	2	57	33	4	
Leaves	1	4	23	67	4	1
Cultivar with white flowers						
Whole flowers	8	8	35	42	4	3
Leaves	2	3	24	68	3	

[a] See Table 2 for identification of pigments
t = trace

two-dimensional NMR techniques (Tables 2 and 3) [1].

The UV–Vis spectrum of **2** taken on-line during HPLC showed a visible maximum at 532 nm with A_{440}/A_{532} and A_{278}/A_{532} of 22% and 90%, respectively, indicating the presence of an anthocyanidin 3-glycoside with an aromatic acyl group. The relative high mobility in both aqueous and alcoholic TLC systems and long retention time (HPLC) of **2** compare to pigment **1** (Table 2), confirmed aromatic acylation. Pigment **2** was found to contain the same aglycone (delphinidin) and sugar (β-galactopyranoside) as **1** (Table 3 and 4), however, the SEFT NMR spectrum of **2** showed in addition to the corresponding signals of **1**, four positive and one negative carbon signals (Table 4). Two of these signals (δ 146.3 and 110.5) each represented two carbon atoms. The latter signal was correlated (HSC) with a 2H singlet at δ 7.07, in accordance with a galloyl (3,4,5-trihydroxybenzoyl) moiety. The pronounced downfield shift of H-2″ (1.6 ppm) compared to the analogous H-2″ signal of **1** and **3** (Table 3), showed that the galloyl moiety was connected to C-2″ on the galactose ring. This linkage was also confirmed by the observation that H-1″ and H-3″ of **2** were ca 0.3 ppm more deshielded than the corresponding signals of **1** and **3** (Table 3). Similarly, C-2″ was deshielded (more than 1 ppm) and C-1″ and C-3″ were shielded (more than 1.6 ppm) compared to the corresponding signals of **1** and **3** (Table 4). A molecular ion $[M+H]^+$ at *m/z* 617 confirmed the structure of **2** to be delphinidin 3-*O*-(2″-*O*-galloyl-β-galactopyranoside). This anthocyanin has previously been found only in two species belonging to the genus *Victoria* [4].

The UV–Vis spectrum of pigment **5** taken on-line during HPLC showed a visible maximum at 528 nm with A_{440}/A_{528} of 24.8%, indicating the presence of an anthocyanidin 3-glycoside with extended chromophore. The UV-vis spectrum of **5** also showed higher absorbance around 278 nm (A_{278}/A_{528} = 90%) than those of delphinidin 3-galactoside (A_{278}/A_{527} = 69%), indicating the presence of an aromatic acyl group. The downfield part of the 1D ^{1}H NMR spectrum of **5** included a 3H ABX system at δ 8.08 (H-6′), δ 7.88 (H-2′) and δ 6.87 (H-5′) and a 3H ABX system at δ 6.94 (H-8), δ 6.75 (H-6) and δ 9.06 (H-4) (Table 3). The corresponding aglycone carbons were assigned by the one-bonding heteronuclear shift correlation (HSC) experiment, and the signals of the quaternary carbon atoms (Table 4) were found by a heteronuclear coupling modulated spin echo (SEFT) experiment to be in accordance with a cyanidin aglycone [5]. The cross-peaks in the long-range heteronuclear NMR experiment (HMBC) at 9.06/164.5 ppm (H-4/C-2), 9.06/145.5 ppm (H-4/C-3), 6.87/120.82 ppm (H-5′/C-1′), 6.87/147.5 ppm (H-5′/C-3′), 8.08/156.1 ppm (H-6′/C-4′), 7.08/140.1 ppm (H-2‴,6‴/C-4‴), 7.08/167.8 ppm (H-2‴,6‴/C-7‴), and 2.18/172.8 ppm (H-2⁗/C-1⁗) were of particular value for the assignments of the quaternary carbon atoms. Except for the signals corresponding to the aglycone part of the NMR spectra, the signals of **5** were similar to those of **4**, representing a galactosyl, acetyl and galloyl unit (Table 3 and 4). The pronounced downfield shift of H-2″ (1.7 ppm) in the ^{1}H NMR spectrum of **5** compared to similar signals of **1** (Table 3), showed that one acyl moiety was connected to C-2″ on the sugar. This link-

Table 2. Chromatographic and spectral data of anthocyanins from *Nymphaéa* × *marliacea*; delphinidin 3-*O*-β-galactopyranoside (**1**), delphinidin 3-*O*-(2″-*O*-galloyl-β-galactopyranoside) (**2**), delphinidin 3-*O*-(6″-*O*-acetyl-β-galactopyranoside) (**3**), delphinidin 3-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-β-galactopyranoside) (**4**), cyanidin 3-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-β-galactopyranoside) (**5**)

Compound	TLC (R_f) FHW	BAW	Vis. max. (nm)	On-line HPLC A_{440}/A_{max} (%)	R_t (min)	ES-MS *m/z*
1	0.23	0.17	527	26	12.87	465
2	0.55	0.34	532	22	13.97	617
3	0.30	0.40	532	22	16.67	507
4	0.58	0.52	537	20	17.60	659
5	0.67	0.66	528	25	19.06	643

Table 3. ^{1}H NMR spectral data for delphinidin 3-*O*-*β*-galactopyranoside (**1**), delphinidin 3-*O*-(2″-*O*-galloyl-*β*-galactopyranoside) (**2**), delphinidin 3-*O*-(6″-*O*-acetyl-*β*-galactopyranoside) (**3**), delphinidin 3-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-*β*-galactopyranoside) (**4**), cyanidin 3- *O*-(2″-*O*-galloyl-6″-*O*-acetyl-*β*-galactopyranoside) (**5**) in CD_3OD : CF_3COOD (95 : 5) at 25°

	1 δ (ppm) *J* (Hz)	**2** δ (ppm) *J* (Hz)	**3** δ (ppm) *J* (Hz)	**4** δ (ppm) *J* (Hz)	**5** δ (ppm) *J* (Hz)
Aglycone					
4	9.07 *s*	9.05 *s*(*broad*)	9.00 *d* 0.8	8.99 *d* 0.8	9.06 *s*
6	6.74 *d* 1.9	6.71 *d* 2.0	6.75 *d* 1.9	6.73 *d* 1.9	6.75 *d* 2.0
8	6.95 *s*(*broad*)	6.89 *dd* 2.09, 0.8	6.96 *dd* 1.9, 0.8	6.90 *dd* 0.8, 1.9	6.94 *d* 2.0
2′	7.88 *s*	7.68 *s*	7.87 *s*	7.64 *s*	7.88 *d* 2.5
5′					6.87 *d* 8.7
6′	7.88 *s*	7.68 *s*	7.87 *s*	7.64 *s*	8.08 *dd* 2.5, 8.7
3-*O*-*β*-galactopyranoside					
1″	5.36 *d* 7.8	5.65 *d* 7.9	5.34 *d* 7.7	5.63 *d* 7.9	5.65 *d* 8.0
2″	4.11 *dd* 7.8, 9.7	5.77 *dd* 7.9, 9.9	4.11 *dd* 7.7, 9.6	5.78 *dd* 7.9, 9.9	5.78 *dd* 8.0, 9.9
3″	3.78 *dd* 9.7, 3.7	4.07 *dd* 9.9, 3.4	3.78 *dd* 9.6, 3.4	4.07 *dd* 9.9, 3.7	4.06 *dd* 9.9, 3.4
4″	4.05 *d* 3.7	4.13 *d* 3.4	4.04 *dd* 3.4, 1.0	4.13 *d* 3.7	4.12 *d* 3.4
5″	3.85 *m*	4.02 *ddd* 0.9, 4.8, 7.2	4.15 *ddd* 1.0, 3.7, 8.3	4.27 *dd* 3.5, 8.8	4.29 *dd* 3.3, 8.5
6″A	3.86 *m*	3.97 *dd* 7.2, 11.4	4.43 *dd* 8.3, 11.8	4.52 *dd* 8.5, 11.9	4.51 *dd* 8.5, 12.0
6″B	3.86 *m*	3.92 *dd* 4.8, 11.4	4.37 *dd* 3.7, 11.8	4.44 *dd* 3.4, 11.9	4.45 *dd* 3.3, 12.0
2″-galloyl					
2‴, 6‴		7.07 *s*		7.06 *s*	7.08 *s*
6″-acetyl					
2⁗			2.13 *s*	2.18 *s*	2.18 *s*

age point was also indicated by the observation that H-1″ and H-3″ of **5** were ca 0.3 ppm more deshielded than the corresponding signals of **1** (Table 3). Similarly, C-2″ was deshielded (0.8 ppm) and C-1″ and C-3″ shielded (2 ppm) compared to the corresponding signals of **1** (Table 4). A cross-peak in the HMBC spectrum at 168.1/5.78 ppm between C-7‴ of the galloyl moiety and H-2″ confirmed this linkage. The acetyl group was determined to be linked to the 6″-position of the galactosyl group since the chemical shifts of H-6A″, H-6B″ and C-6″ were shifted 0.6, 0.6, and 2.9 ppm downfield, respectively, C-5″ was shifted 2.6 ppm upfield compared to the corresponding signals of **1** (Table 3 and 4), and because of the cross-peak in the HMBC experiment at 172.8/4.51 ppm between C-1⁗ of the acetyl group and H-6A″. A molecular ion $[M+H]^+$ at *m/z* 643 confirmed the structure of **5** to by cyanidin 3-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-*β*-galactopyranoside), a novel anthocyanin.

The flowers and leaves of three cultivars of *Nymphaéa* × *marliacea* with different flower colours contained the same five anthocyanins (**1**–**5**) (Table 1). The delphinidin derivatives **3** and **4** together constituted more than 75% of the total anthocyanin content in all examined samples. The relative quantity of delphinidin 3-*O*-(6″-*O*-acetyl-*β*-galactopyranoside) (**3**) was higher in the flowers than in the leaves, while this ratio was the opposite for delphinidin 3-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-*β*-galactopyranoside) (**4**). With respect to relative amounts, both flowers and leaves of the red cultivar had more than three times as high content of the red cyanidin derivative (**5**) compared to the other cultivars. Within the flowers of the red cultivar, the red-orange stamens were enriched with respect to **5** in comparison with the red-violet petals which contained greater relative amounts of **4**.

Anthocyanins acylated with benzoic acid derivatives seem to have restricted distribution [6]. Gallic acid (3,4,5-trihydroxybenzoic acid) has for instance been identified only as cyanidin 3-(2″-galloylglucoside) in *Dipteronia sinensis* and *Acer* taxa, as cyanidin 3-(2″-galloylrutinoside) in *Acer* taxa [2, 3], as the 3-(2″-galloylgalactosides) of delphinidin and cyanidin in two *Victoria* (Nymphaéaceae) species [4], in addition to the anthocyanins identified in the cultivars of *Nymphaé* × *marliacea* examined in this paper. The finding of gallic acid as the acyl group of at least one major anthocyanin in all examined Nymphaéaceae samples may thus have chemotaxonomic significance.

EXPERIMENTAL

Plant material

Flowers and leaves of three water lily, *Nymphaéa* × *marliacea*, cultivars with white, pink and red coloured flowers, respectively, were collected during June–September 1996 in the Botanical Garden of the University of Bergen (BG) and frozen (−20°). Samples of the cultivar with red flowers, which belongs to var. Escarboucle, were also collected at

Table 4. ^{13}C NMR spectral data for delphinidin 3-*O*-*β*-galactopyranoside (**1**), delphinidin 3-*O*-(2″-*O*-galloyl-*β*-galactopyranoside) (**2**), delphinidin 3-*O*-(6″-*O*-acetyl-*β*-galactopyranoside) (**3**), delphinidin 3-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-*β*-galactopyranoside) (**4**), cyanidin 3-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-*β*-galactopyranoside) (**5**) in CD_3OD : CF_3COOD (95 : 5) at 25°

	1 *δ* (ppm)	**2** *δ* (ppm)	**3** *δ* (ppm)	**4** *δ* (ppm)	**5** *δ* (ppm)
Aglycone					
2	164.49	164.50	164.38	164.51	164.47
3	145.96*	145.44*	145.98*	145.62*	145.46
4	136.61	136.25	135.68	135.34	135.72
5	159.03†	159.25†	159.38†	159.12†	159.44†
6	103.29	103.26	103.30	103.30	103.35
7	170.38	170.42	170.24	170.33	170.40
8	95.03	95.06	95.13	95.18	95.24
9	157.72†	157.67†	157.64†	157.66†	157.69†
10	113.29	113.12	113.87	112.97	113.03
1′	120.07	119.69	120.01	119.53	120.82
2′	112.62	112.62	112.71	112.68	117.80
3′	147.56	147.38	147.57	147.38	147.52
4′	144.71*	144.71*	144.91*	144.79*	156.05
5′	147.56	147.38	147.57	147.38	117.41
6′	112.62	112.62	112.71	112.68	128.77
3-*O*-*β*-galactopyranoside					
1″	104.63	102.80	104.06	102.41	102.00
2″	72.16	73.42	71.89	73.20	72.91
3″	74.87	73.02	74.60	72.74	72.85
4″	70.14	70.33	70.31	70.48	70.56
5″	77.80	78.10	75.15	75.46	75.51
6″	62.35	62.34	65.20	65.17	65.27
2″-galloyl					
1‴		120.83		120.82	121.06
2‴,6‴		110.48		110.50	110.52
3‴,5‴		146.33		146.30	146.37
4‴		140.14		140.15	140.07
7‴		168.11		168.14	167.75
6″-acetyl					
1⁗			172.76	172.83	172.80
2⁗			20.69	20.74	20.75

*,† Assignments with the same superscript may be reversed.

ARBOHA, Minde, Bergen. Voucher specimens have been deposited in BG.

Isolation of pigments

The samples were cut into pieces and extracted with 0.5 M citric acid in MeOH at 5° C. The filtered extract of the red flowers of var. Escarboucle was concd under red. pres., purified by partition against hexane and ethyl acetate and applied to an Amberlite XAD-7 column [7]. The anthocyanins in this purified extract were separated by prep. HPLC according to published procedure [1]. See Ref. 1 for more details. The identification of the anthocyanins in the other extracts were based on UV–vis spectroscopy and co-chromatography (HPLC).

Analytical chromatography

TLC was carried out on microcrystalline cellulose (F1440, Schleicher and Schüll) with the solvents BAW (1-butanol–HOAc–H_2O; 4 : 1 : 5, upper phase) and FHW (HCO_2H–conc. HCl–H_2O; 1 : 1 : 2). Analyt. HPLC was performed with a ODS-Hypersil column (20 × 0.5 cm, 5 *μ*m) using isocratic elution (90% A, 10% B) in 4 min, linear gradient from 10% B to 100% B during the next 17 min, isocratic elution (100% B) in 4 min followed by linear gradient from 100% B to 10% B over 1 min. The flow rate was 1.0 ml min^{-1}, and aliquots of 15 *μ*l were injected.

Spectroscopy

UV–vis absorption spectra were recorded on-line during HPLC analysis using a photodiode array detector (HP 1050, Hewlett-Packard). Spectral measurements were made over the wavelength range 240–600 nm in steps of 2 nm. The relative quantitative data were based on the average values of the absorptions on every second nm between 500 and 540 nm, without

taking into account the different molar absorption coefficients of the pigments. The NMR experiments (DQF-COSY, HMBC, HSC, SEFT) were obtained at 600.13 MHz and 150.92 MHz for ^{1}H and ^{13}C respectively, on a Bruker DRX-600 instrument at 25°. The deuteriomethyl ^{13}C signal and the residual ^{1}H signal of the solvent (CF_3CO_2D–CD_3OD; 5:95, v/v) were used as secondary references (δ 49.0 and δ 3.4 from TMS, respectively). See Ref. 1 for more experimental details. The mass spectra were obtained on a Quattro II MS/MS (Micromass, U.K.) by flow injection into the electrospray source. The instrument was operated in the positive ion mode, and the mobile phase carrier was a methanol–water (50:50) mixture containing 0.1% formic acid. The carrier was pumped into the source at a flow rate of 100 μl min^{-1}. The samples were dissolved in 3% formic acid (in methanol) prior to analysis.

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